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Award Number: W81XWH-12-1-0436

TITLE: TrkB Activators for the Treatment of Traumatic Vision Loss

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REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	30Septmeber2012-29September2013

4. TITLE AND SUBTITLE
TrkB Activators for the Treatment of Traumatic Vision Loss

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0436

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Emory University

Atlanta, GA 30322

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Pressure waves due to explosions can damage the neurons of the eye and visual centers in the brain, leading to functional loss of vision. There are currently few treatments for such injuries that can be deployed rapidly in the field to mitigate such damage. Our research team is developing small molecule activators of TrkB, the cognate receptor for brain-derived neurotrophic factor (BDNF), which can be administered systemically and cross the blood brain/retina barrier (BBB). In the initial year of this grant, we have developed procedures for damaging the optic nerve and initiated experiments to establish assays for measuring retinal ganglion cell (RGC) loss after optic nerve damage. We developed a novel, rapid assay for ganglion cell death using microplate fluorescence measurements. The time course of retinal ganglion cell loss after optic nerve damage was established, demonstrating that most cell loss occurs during the first six days after trauma. We have developed a new synthesis for HIOC, one of our lead TrkB activators. Drug tests have been initiated, but thus far we have not observed consistent protection from optic nerve crush. We are altering test parameters (dose, timing, administration route) to further assess the utility of the drugs for improving retinal ganglion cell survival after optic nerve crush. We have begun construction of a blast cannon and will begin testing soon.

15. SUBJECT TERMS
trauma, neuroprotection, retina, optic nerve, TrkB

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Pressure waves due to explosions can damage the neurons of the eye and visual centers in the brain, leading to functional loss of vision. There are currently few treatments for such injuries that can be deployed rapidly in the field to mitigate such damage. Our research team is developing small molecule activators of TrkB, the cognate receptor for brain-derived neurotrophic factor (BDNF). BDNF has been shown to have neuroprotective effects in a number of degeneration models, including optic nerve crush and bright light-induced retinal degeneration (Gauthier et al., 2005; Weber et al., 2010). However, BDNF must be injected intraocularly or into the brain to be effective, as it does not cross the blood brain/retina barrier (BBB), making it impractical to deploy in the field. In contrast, the compounds we are developing can be administered systemically and readily cross the BBB (Jang et al., 2010a,b,c). Following peripheral injection, the drugs activate TrkB receptors in the retina and the brain, and appear to show no systemic toxicity. In preliminary studies, we have shown that they protect against light-induced retinal degeneration (Shen et al., 2012). The goal of this project is to develop effective treatments for traumatic blast-related retinal and visual system damage that can be delivered on the battlefield. We hypothesize that small molecule activators of TrkB will be useful for this purpose. We proposed 3 specific aims to test this hypothesis, investigating the utility of TrkB activators to prevent retinal ganglion cell death following optic nerve crush, protect retinal cells from blast-induced injury to the eye, and protect central visual pathways from traumatic blast-induced injury.

BODY

The statement of work for year 1 was to use the optic nerve crush model to test the effects of at least two small molecule TrkB activators. In order to do this we needed to establish the optic nerve injury model in our lab and develop assays for retinal ganglion cell death.

Experiments were initiated to establish assays for measuring retinal ganglion cell (RGC) loss after optic nerve crush. Two approaches were taken. One was to count Brn3a immunoreactive cells in retinal whole mounts. Brn3a is a specific marker for retinal ganglion cells (Nadal-Nicolas et al., 2009); it is expressed by approximately 90% of ganglion cells. The other approach was to measure fluorescence of retinal extracts of Thy1-CFP mice, which express cyan fluorescent protein in retinal ganglion cells (Feng et al., 2000).

Immunofluorescent Staining of Retinal Ganglion Cells - We have optimized an immunostaining protocol that allows for detection of retinal ganglion cells in retinal flatmounts. The majority of RGCs can be stained with antibodies against the RGC marker Brn-3a and visualized by fluorescent microscopy. Brn-3a also has the advantage of being a nuclear marker, so cell bodies can be easily identified and counted. This has been useful for examining the RGC damage and death time course after optic nerve crush (Figure 1).

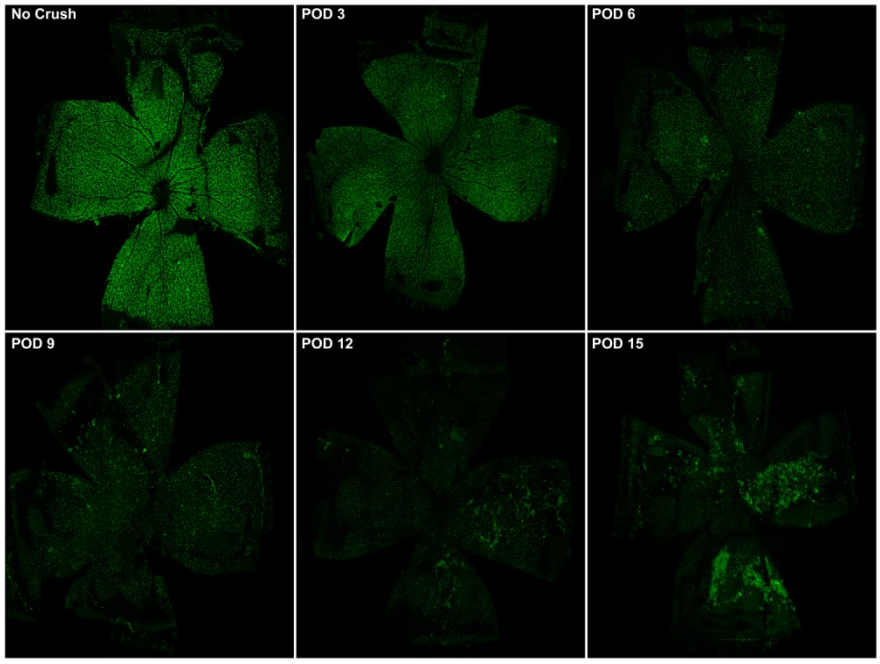

Figure 1. Time course of RGC signal following optic nerve crush. Confocal images of retinal flatmounts were taken every three days over a 15 day period to show the progression of RGC loss after optic nerve crush. POD = post-operation day.

As can be seen in Figure 1, maximal retinal ganglion cell loss occurs approximately 6 days after optic nerve crush.

Measurement of Fluorescence of Thy1-CFP ganglion cells. Our initial studies assessing retinal ganglion cell death following optic nerve crush involved counting Brn3a positive ganglion cells, labeled immunocytochemically, in retinal whole mounts. The procedure is extremely time consuming, taking approximately one week to complete the analysis following euthanizing the mice. The new procedure involved the use of transgenic Thy1-CFP mice, whose retinal ganglion cells express cyan fluorescent protein, and measuring fluorescence of retinal supernatant fractions using a microplate reader. Briefly, optic nerve crush surgery was performed on the right eye, with the left eye serving as the uncrushed control. The mice were euthanized 6 days thereafter. Retinas were immediately dissected and frozen. Retinal homogenates were prepared in RIPA buffer with a protease inhibitor cocktail. Retinal homogenates were centrifuged for 10 minutes. Each supernatant fraction was plated in triplicate on a black 96-well plate and the relative levels of fluorescence were detected by a BioTek H1 plate reader. In a separate cohort of mice, Brn3a positive retinal ganglion cells were counted. As seen in Figure 1, the percentage decrease in Brn3a positive cells following optic nerve crush was nearly identical to the decrease in CFP fluorescence measured in the plate reader. A number of control experiments were performed to further validate the assay (not shown). The new assay takes only hours, as opposed to days for counting the Brn3a positive cells. The rate-limiting factor is breeding and genotyping the Thy1-CFP mice. This work was presented at the International Society for Eye Research (ISER) Molecular Mechanisms of Glaucoma Symposium. A manuscript is being prepared for submission as a technical brief.

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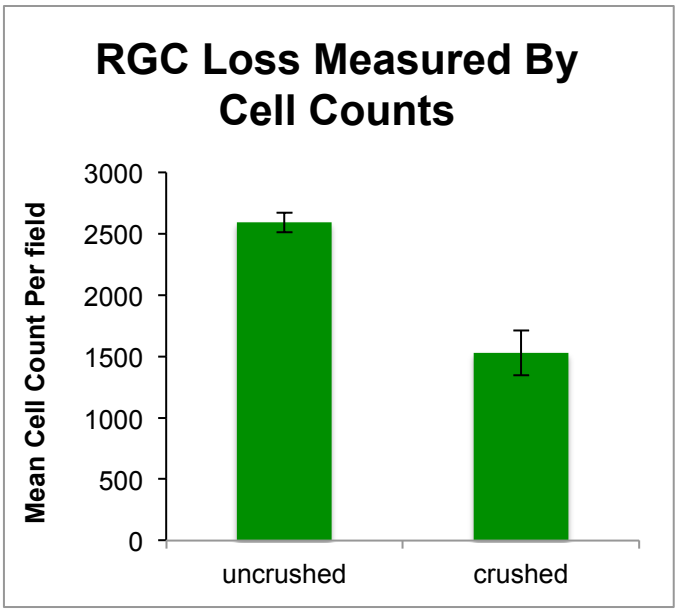

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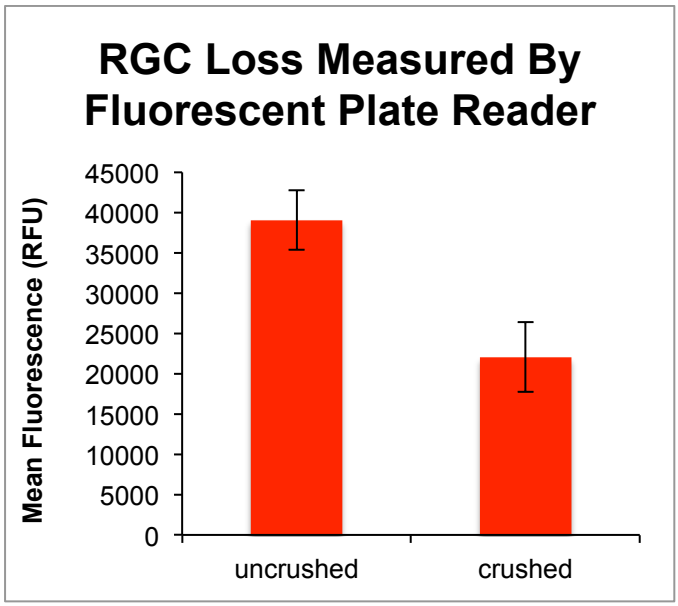

Figure 2. Optic nerve crush was performed on the right eye of adult mice, which were sacrificed 6 days after surgery. Cell counts of immunostained retinal flatmount images (n=3) show a 41% loss of cells after crush (A). Fluorescent reads of retinal homogenates (n=4) show a 44% loss of signal following crush (B).

Evaluating different methods of optic nerve injury. In order to detect drug effects with our optic nerve crush model, we must carefully consider how much ocular damage is appropriate for our studies. If our optic nerve crush is too damaging to the RGC axons, we might mask the effects of a protective drug, simply because its capacity to rescue cells is overwhelmed by the level of damage induced by crush. In this experiment, we sought to test different “severities” of optic nerve crush to see if it was possible to develop a less severe insult to the optic nerve.

Optic nerve crush surgeries were performed on the right eyes of ten mice. The fellow eye was not operated on and served as a control. Five mice received a crush with flat-tip forceps that squeezed all along the sides of the optic nerve. The other five mice received a crush with angled-tip forceps that only partially squeezed the sides of the optic nerve. Crush performed with flat-tip forceps were considered “severe”, while those performed with angled-tip forceps were considered “mild.”

As anticipated, the mild crush delivered by angled forceps exhibited higher cell counts than flat forceps two weeks after optic nerve crush. This was observed in both central (Figure 3) and peripheral retina (data not shown) images. But while cell counts trended towards higher values for the mild crush, it was also associated with more variability. This variance is absent in the severe crush because so little of the RGC population survives in the weeks following the procedure. Also, the angled forceps are more difficult to place on the optic nerve since there is only one small contact point, as opposed to the flat forceps which physically touch all along the end of the instrument. Small differences in the way that the forceps were held could have contributed to the variability seen with the mild crush. Overall, the mild crush delivers just enough force to cause injury to the RGC axons and may be a favorable method for our drug testing in the future, provided that we control for the variability associated this method.

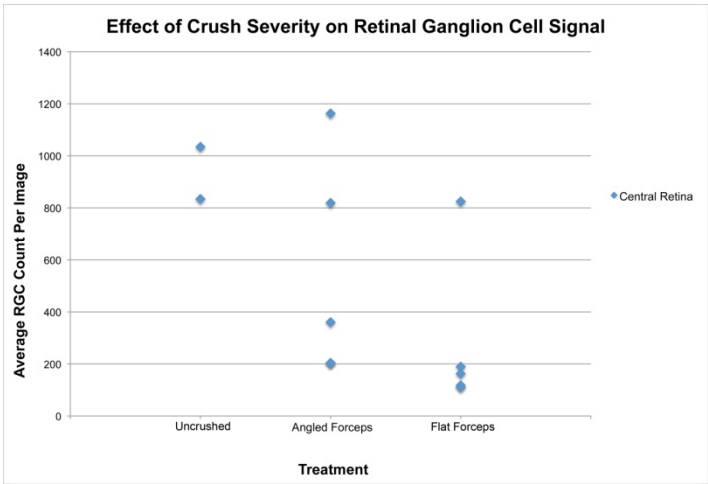

Figure 3. Effect of angled-tip (mild crush) or flat-tip (severe crush) forceps on RGC counts 14 days after crush surgery. Mean cell counts of central retina images were determined by Cell Profiler image analysis software. For each forceps group n=5, for uncrushed n=2.

After comparing RGC outcomes with mild and severe crush procedures, we wanted to pursue future experiments with a mild crush as it left a larger proportion of RGCs alive. However, the inherent variability in RGC counts when using angled forceps could confound potential drug effects. In the following experiment, we tested a microvascular clip as an alternative to the angled forceps. The clip could deliver a less severe crush, but also deliver a set pressure each time it was used. This experiment, tested whether a microvascular clip could cause a mild crush with reduced variability as compared to forceps. Optic nerve crush surgeries were performed on the right eyes of four mice using the microvascular clip. The fellow eye was not operated on and served as a control. Two more animals received an optic nerve crush with forceps. (Cell counts from the forceps crush group were later pooled with previous forceps crush data to confirm that the counts were consistent.) Mice were sacrificed 14 days after surgery. Retinas were collected and immunostained for Brn-3a, imaged, and analyzed with Cell Profiler software.
The microvascular clip crush eyes exhibited mean cell counts that were more than 50 percent of the uncrushed value, while forceps crush eyes were only 12 percent of control (Figure 4). While the microvascular clip successfully delivered a mild crush, it did not resolve the issue of variable cell counts. The cell counts still spanned a range similar to that seen with angled forceps. We will continue testing ways to deliver a consistent mild crush.

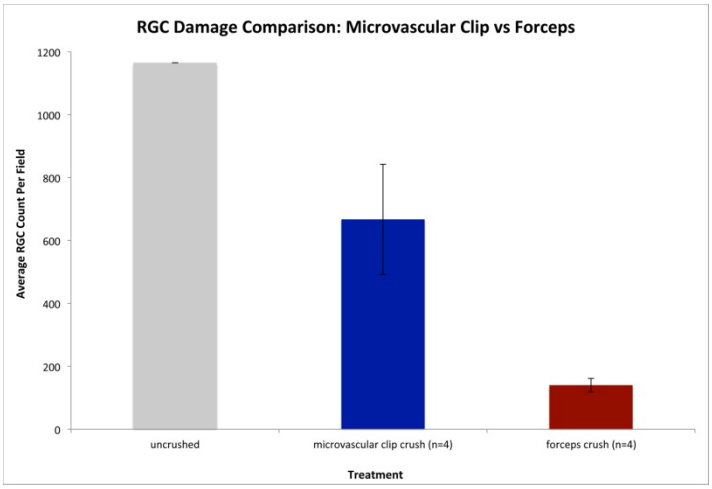

Figure 4. Mean RGC counts per image for optic nerve crushes delivered by microvascular clip or forceps. Cell counts obtained by Cell Profiler image analysis software. Crushes with the microvascular clip resulted in smaller decreases in RGC signal over 14 days, but with more variability than images from crushes delivered by forceps.

Effect of HIOC on RGC Survival after Optic Nerve Crush

We continued to use the severe optic nerve crush procedure in our initial test of TrkB activators in order to test efficacy against a severe injury and because of the variability associated with the less severe injury. In this experiment we tested N-[2-(5-hydroxy- 1H-indol-3-yl)ethyl]-2-oxopiperidine-3-carboxamide (HIOC), in the optic nerve crush model. As RGCs possess TrkB receptors, we hypothesize that administration of HIOC immediately around the time of optic nerve crush would allow more RGCs to survive the injury.

THY1-CFP mice were injected i.p. with 40 mg/kg HIOC or vehicle (20% DMSO in PBS) roughly one hour before receiving the optic nerve crush or transection procedure. Under anesthesia, each mouse had the right optic nerve crushed. The fellow eye was left as a control. Mice were allowed to recover and were injected once daily with HIOC or vehicle following surgery until sacrifice at post-op day 6. Retinas were immediately dissected and frozen. Retinal homogenates were

prepared in RIPA buffer with a protease inhibitor cocktail. The relative levels of fluorescence were measured in the supernatant fractions.

The mean fluorescence from naïve retinas (in regular fluorescence units) was 37502 ± 937 (Figure 5). The mean fluorescence of crushed/transected vehicle retinas was 18564 ± 567 and that of HIOC-treated retinas was 18338 ± 826. We did not see protection associated with HIOC treatment. It is possible that the mice are metabolizing the drug too quickly to allow for sufficient TrkB activation or the dose was insufficient. We are currently testing alternative delivery methods, including the use of osmotic minipumps.

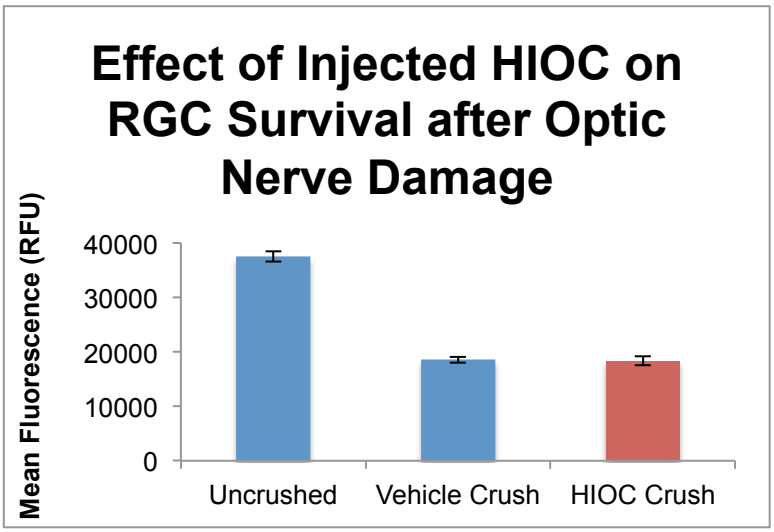

Figure 5. THY1-CFP mice received intraperitoneal injections of either 40 mg/kg HIOC or vehicle (20% DMSO in PBS) prior to receiving an optic nerve crush or transection to the right eye. Injections continued until 6 days after surgery, when mice were sacrificed. Retinas were homogenized and the fluorescence from each retina was quantified using a fluorescent plate reader.

We performed a dose response experiment and administered three different doses of HIOC to see if RGC protection corresponded with dosage strength. Twelve transgenic THY1-CFP mice of roughly 12 weeks of age received an optic nerve crush to the right eye with forceps. The fellow eye was not operated on and served as a control. One hour before each surgery, the mice received an intraperitoneal injection of vehicle (20 percent DMSO in PBS) or 10, 40, or 100 mg/kg of HIOC in vehicle. Injections were also given once daily for the first two days following surgery. Six days after surgery, mice were sacrificed, and retinas were collected and immediately frozen on dry ice. Retinas were homogenized and fluorescence was quantified.

The vehicle-treated crush mouse experienced a loss of roughly half its RGC signal (Figure 6). However, this same trend also applied to the HIOC-treated mice, which experienced large reductions in their RGC signal as well. It is possible that this crush method was too severe to detect protection by HIOC. Alternatively, pharmacokinetic factors may have prevented a neuroprotective action of the drug.

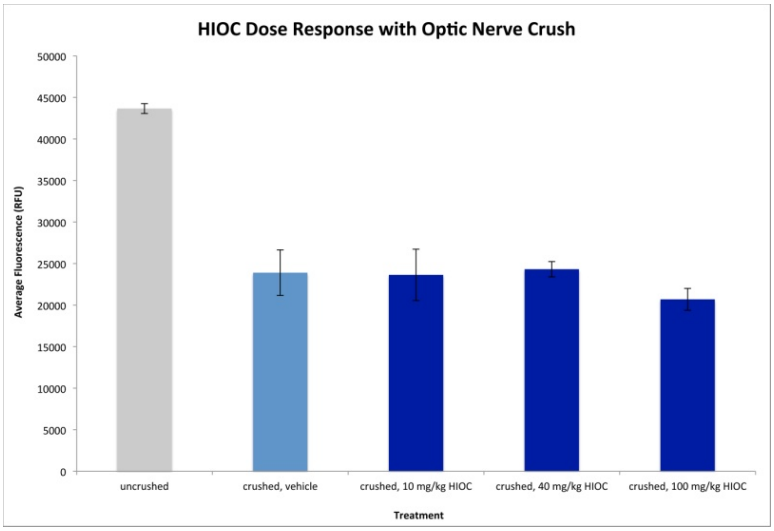

Figure 6. Average fluorescence readings from THY1-CFP retina homogenates as measured by a fluorescent plate reader. HIOC treatment did not protect RGCs from damage as compared to vehicle.

To address the potential pharmacokinetic issue, we initiated the osmotic minipump experiments described in the proposal. C57BL6/J mice were pretreated with an intraperitoneal injection of HIOC (40 mg/kg) or vehicle, anesthetized and subjected to unilateral optic nerve crush. Each mouse then received a subcutaneous implant of an Alzet Model 2001 osmotic minipump containing HIOC or vehicle (40% DMSO). The HIOC concentration used provided a calculated drug delivery rate of 20 mg/kg/day. After six days, the mice were euthanized and the numbers of Brn3a-immunoreactive retinal

ganglion cells (RGC) were determined in retinal flat mounts. Optic nerve crush caused a significant reduction in RGC number in both the vehicle and HIOC-treated mice ($p<0.01$, Figure 7). While the RGC number was slightly higher in the crushed HIOC-treated eye than in the crushed vehicle-treated eye, the effect was not statistically significant. We are currently testing additional doses of HIOC.

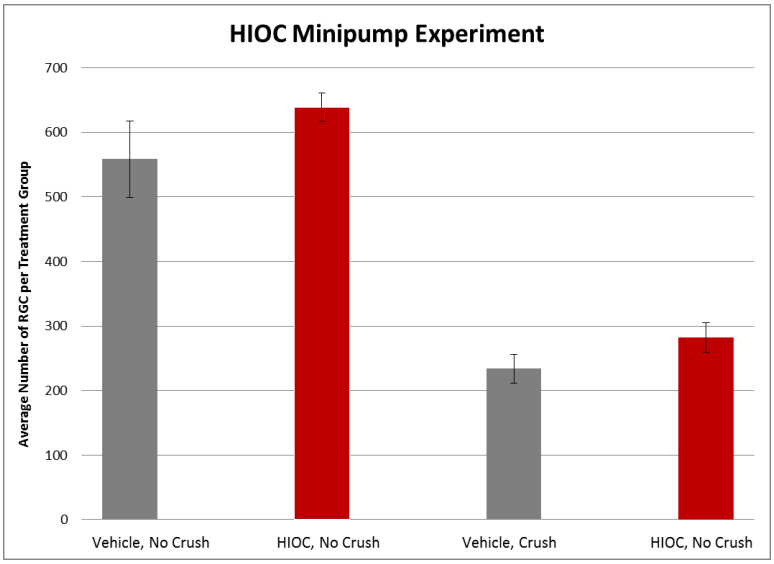

Figure 7. Effect of HIOC on optic nerve crush-induced retinal ganglion cell death. HIOC or vehicle were deliver via Alzet osmotic minipumps. Nerve crush significantly reduced RGC number ($p<0.01$), but HIOC did not significantly protect the cells from injury.

Effects of TrkB agonist Deoxygedunin

Deoxygedunin is a naturally occurring compound that has been shown to activate TrkB receptors in the mouse brain (Jang et al., PLoS One 2010; 5(7):e11528). We sought to test the effectiveness of deoxygedunin in protecting RGCs against apoptosis following optic nerve crush. Five C57BL/6J mice from Jackson Laboratories were given an intraperitoneal injection of 5 mg/kg deoxygedunin and 4 were given vehicle (34% DMSO in PBS). Pharmacokinetic studies have shown that deoxygedunin is active two hours after administration. Therefore, injections were staggered so that each mouse received the optic nerve crush two hours after the initial injection. The right eye was operated on with the left eye serving as a control. Injections of deoxygedunin or vehicle were given daily until 6 days post-surgery when the mice were sacrificed. Eyes were enucleated, and retinas immunostained for the RGC marker Brn-3a. The cell counts from vehicle and deoxygedunin-treated eyes that received crush decreased by 41% and 38.2%, respectively when compared to uncrushed eyes (Figure 8, $p<0.01$). We did not detect a protective drug effect with deoxygedunin at the dose tested. As with our other drug studies, we may need to investigate the pharmacokinetics of this agent to see if our dosing regimen needs to be altered in order to provide protection.

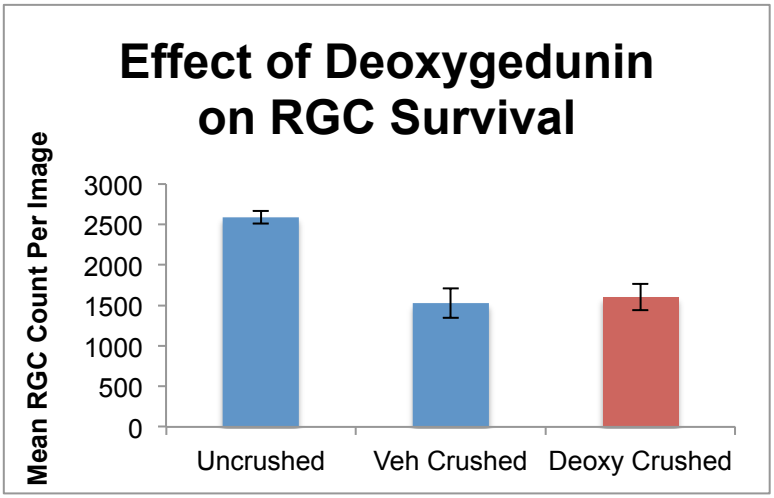

Figure 8. Mice received intraperitoneal injections of either 5 mg/kg deoxygedunin or vehicle (34% DMSO in PBS) prior to receiving an optic nerve crush to the right eye. Injections continued until 6 days after surgery, when mice were sacrificed. Retinas were immunostained for the RGC marker Brn-3a and imaged with a confocal microscope. RGCs were quantified using Cell Profiler software. Nerve crush significantly reduced the number of RGCs ($p<0.01$). Deoxygedunin did not significantly prevent cell loss.

KEY RESEARCH ACCOMPLISHMENTS

- Experiments were initiated to establish assays for measuring retinal ganglion cell death after optic nerve crush. A novel assay of RGC cell death was developed.
- The time course of retinal ganglion cell death was established.
- Experiments were conducted to establish the best techniques for producing optic nerve injury.
- HIOC was synthesized.

- HIOC and Deoxygedunin were tested for neuroproterctive effects in optic nerve crush.
- Construction began on the blast cannon.

REPORTABLE OUTCOMES

An abstract was submitted and a presentation made at the International Society for Eye Research (ISER) Symposium on Molecular Mechanisms in Glaucoma on the new RGC cell death assay. A copy of the abstract from the abstract book is included in the Appendix.

CONCLUSION

We established methods to assess retinal ganglion cell death following optic nerve injury. We have initiated tests of two TrkB activators for neuroprotection in the optic nerve crush model. Thus far, the drugs have been ineffective in protecting retinal ganglion cells from nerve crush-induced cell death. Studies are underway to optimize potential protective effects of the drugs, and plans include testing the drugs for protection against a less severe optic nerve injury and blast-induced retinal injury. We will also test other TrkB activators under development. We may also test other classes of drugs for neuroprotection; if we do so, we will seek approval of a modification of the Statement of Work.

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Poster Abstract 15 – INHIBITORY ACTION OF HYDROGEN SULFIDE DONOR, ACS 67 ON SYMPATHETIC NEUROTRANSMITTER RELEASE IN ISOLATED BOVINE IRIS-CILIARY BODIES

CATHERINE OPERE[1], Ankita Salvi[1], Pratik Bankhele[1], Jamal Jamil[1], Ya Fatou Njie-Mbye[2], Madhura Chitnis[2], Sunny Ohia[2]

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There is evidence that hydrogen sulfide (H_2S) regulates sympathetic neurotransmitter release in mammalian anterior uvea (Kulkarni et al., Neurochem Res. 34:400-6, 2009). However, the effect of the H2S donor-latanoprost hybrid, ACS 67 on sympathetic neurotransmission has not been completely elucidated. In the present study, we investigated the effect of, ACS 67 on electrically evoked [^{3}H]NE release in superfused, bovine iris-ciliary bodies (ICB), in vitro. Isolated bovine ICB were incubated in oxygenated Krebs solution containing 2.5μCi/ml of [^{3}H]NE and then prepared for neurotransmitter release using the superfusion method. Release of [^{3}H]NE was elicited by two (S1 and S2) electrical pulses (300 d.c electrical pulses) applied 27 min apart. In the concentration range, 0.1n^M to 30μ^M, ACS 67 and latanoprost (positive control) attenuated field-stimulated [^{3}H]NE release in isolated bovine ICB in a concentration-dependent manner without affecting basal tritium overflow. At an equimolar concentration of 10 μ^M, the rank order of potency was as follows: ACS 67 (37.3%; $p<0.001$) > latanoprost (29.5%; $p<0.001$) on the neurotransmitter release. Although cystathionine β-synthase inhibitor, aminooxyacetic acid (3 m^M) and the ATP-sensitive potassium channel (KATP) inhibitor, glibenclamide (300μ^M) had no effect ($p>0.05$) on [^{3}H]NE release, they both reversed the inhibitory action of ACS 67 (0.1 to 1 μ^M) on the neurotransmitter release. Moreover, the cyclooxygenase enzyme inhibitor, flurbiprofen (3μ^M) reversed the effect of ACS 67 (10μ^M) on field-stimulated [^{3}H]NE release. In conclusion, the in situ release of H_2S, prostanoids and activation of K_{ATP}-channels contribute to the inhibitory action of ACS 67 on sympathetic neurotransmitter release in isolated bovine ICB.

Poster Abstract 16 – NOVEL METHOD FOR DETERMINING RETINAL GANGLION CELL LOSS

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The retinal ganglion cell (RGC) population is the main cell type that is damaged in glaucoma and other optic neuropathies. Current methods quantify RGC loss by calculating cell densities in images of immunostained retina flatmounts. Here we present a method to rapidly measure quantitative differences in mouse retinal ganglion cells using transgenic THY1-CFP mice and a fluorescent microplate reader. Mice expressing cyan fluorescent protein (CFP) driven by the THY1 promoter and control (background) C57BL/6J mice were obtained from Jackson Laboratories. Optic nerve crush was performed unilaterally in each mouse with the fellow eye serving as a control. Two weeks after crush, retinas from THY1-CFP mice were individually homogenized in RIPA buffer with protease inhibitors and homogenates plated into a black 96-well plate. A fluorescent microplate reader (Synergy H1 model, BioTek) was used to measure the average fluorescence per well. Retinal flatmounts of C57BL/6J mice were immunostained for RGC-selective antigens (THY1 or Brn3A) and immunopositive cells counted. Average fluorescence readings (± SEM) for uncrushed and crushed retinas were 49545 ± 1670 and 15175 ± 739 regular fluorescence units. This corresponds to a 69.4% decrease in RGC signal in the eyes that received the optic nerve crush procedure as compared to control eyes. A similar decrease of 62.9% was found in RGC-specific immunopositive cell counts of C57BL/6J flatmounted retinas. Given these comparable outcomes, we conclude that the fluorescent plate-reader protocol provides the same quantitative data as conventional methods but with considerably less time and effort, as imaging and cell counting is avoided. This method could be used as a quick, informative measurement of the progression of RGC loss following optic nerve injury. Supported by USAMRAA DOD W81XWH-12-1-0436, NIH R01 EY14026, NIH P30 EY06360, Research to Prevent Blindness (RPB) and the Katz Foundation. COI/Financial Disclosure: None.